The Calyculaglycosides: Dilophol-Type Diterpene Glycosides Exhibiting Antiinflammatory Activity from the Caribbean Gorgonian *Eunicea sp.*[1,2]

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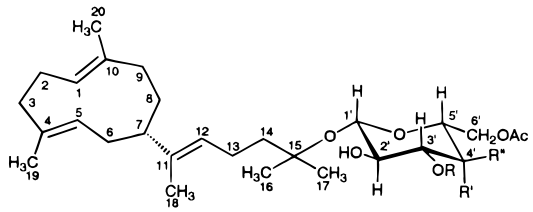
Received March 14, 1997[⊗]

Three new diterpenoid hexose-glycosides, calyculaglycosides A−C (**1**−**3**) were isolated from the Caribbean gorgonian *Eunicea sp.* Calyculaglycosides A−C are rare diterpene glycosides possessing dilophol (**4**) aglycones related in biosynthetic origin to the elemene-type glycoside class of potent antiinflammatory agents known as fuscosides. The structures of the new compounds, which were assigned on the basis of spectral studies, were further corroborated by molecular modeling studies. Calyculaglycoside B (**2**) is an effective topical antiinflammatory agent stronger in potency than the industrial standard indomethacin. Calyculaglycoside B inhibits the synthesis of both prostaglandin PGE_2 and leukotriene LTB_4, suggesting it is a nonselective inhibitor of the 5-lipoxygenase and cyclooxygenase pathways. At concentrations of 10^{-4}−10^{-5}M, calyculaglycoside B produced LC_{50}-level differential responses against a majority of the NCI ovarian cancer lines and several of the renal, prostate, and colon tumor lines.

Introduction

Caribbean octocorals of the order Gorgonacea (sea whips, sea plumes, and sea fans) produce an extensive range of structurally interesting terpenoid metabolites.[3] While the endogenous function of these natural products is of primary interest, an equally critical question is whether these compounds possess selective activities that may afford them a biomedical potential. Gorgonians of the genera *Eunicea* and *Pseudopterogorgia* are among the most abundant octocorals.[4] Research by the Fenical group[5] aimed at the isolation of antiinflammatory agents from these animals led to the discovery of several structurally novel diterpene glycosides. The pseudopterosins[6] and the seco-pseudopterosins[7] are potent antiinflammatory pentose-glycosides possessing aglycones of the serrulatane class of diterpenoids isolated mainly from *Pseudopterogorgia elisabethae.*[8] On the other hand, the fuscosides[9] (*i.e.* **6**), isolated from specimens of *Eunicea fusca*, are antiinflammatory arabinose glycosides possessing fuscol (**5**) aglycones.[10,11]

In this paper, we report the structures of three new diterpenoid glycosides, calyculaglycosides A−C (**1**−**3**), isolated from an undescribed gorgonian species of the genus *Eunicea*. Calyculaglycosides A−C are glycosides possessing dilophol (**4**) aglycones.[12] Natural products possessing a cyclodecane framework are frequently found as germacrane sesquiterpenoids in terrestrial plants.[13] On the other hand, diterpenoids having a 10-membered ring are much rarer. Since the discovery of dilophol other diterpenoids having a 10-membered ring have been obtained from marine resources, including several Caribbean gorgonian species of the genus *Eunicea*.[14a,b] However, calyculaglycosides A−C (**1**−**3**) are the first glycosides possessing the dilophol diterpenoid carbon skeleton.



Calyculaglycoside A (**1**) R= Ac; R'= OH; R"= H

Calyculaglycoside B (**2**) R= H; R'= H; R"= OAc

Calyculaglycoside C (**3**) R= H; R'= OAc; R"= H

Freshly collected specimens of *Eunicea sp.*[15,16] were extracted with 1:1 $CHCl_3$−MeOH. Size-exclusion chro-

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⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

(1) Taken in part from the Ph.D. Dissertation of O. M. Cóbar, University of Puerto Rico, 1996.

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S0022-3263(97)00472-6 CCC: $14.00

Table 1. ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, NOESY, and HMBC Spectral Data for Calyculaglycoside B (2)

position	δ_H, mult, intrg, (J in Hz)[a]	δ_C (mult)[b]	^{1}H−^{1}H COSY[c]	NOE[c]	HMBC[d]
1	4.93, br t, 1H, (6.6)	125.7 (d)	H2, Me20	H2, H5, H9	H9
2	2.14, m, 2H	24.7 (t)	H1	H1, Me20	H1
3	2.08, m, 2H	38.8 (t)		H5, Me19	H5
4	−	133.5 (s)			H6α, H6β
5	5.07, br t, 1H, (6.6)	125.8 (d)	H6α, H6β, Me19	H1, H3, H6β, H7	H6α, H6β
6α	2.14, m, 1H	28.2 (t)	H5, H6β, H7	H6β, H8α, Me19	H5
6β	1.87, m, 1H (7.5, 7.6, 15.0)		H5, H6α, H7	H5, H6α	
7	1.46, m, 1H (3.4, 3.4, 7.2, 7.2)	47.7 (d)	H6αβ, H8αβ, Me18	H5	H5, H6αβ, H8αβ, Me18
8α	1.24, m, 1H (6.2, 6.2, 13.3)	28.3 (t)	H7, H8β, H9	H6α, H8β	H6α, H6β, Me16, Me17
8β	1.62,m, 1H		H7, H8α, H9	H8α	
9	2.00, m, 2H	39.4 (t)	H8α, H8β	H1, H2	H1
10	−	133.1 (s)			H8α, H8β, H9
11	−	134.1 (s)			H7, H8α, H8β, Me18
12	5.00, br t, 1H, (6.9)	125.1 (d)	H13, Me18		H7, Me18
13	2.10, m, 2H	24.0 (t)	H12	Me16, Me17	H12, Me18
14	2.00, m, 2H	37.8 (t)		Me16, Me17	H12
15	−	81.6 (s)			H1′
Me_{16}	1.22, s, 3H	24.0 (q)[e]		H1′, H13, H14	
Me_{17}	1.22, s, 3H	24.5 (q)[e]		H1′, H13, H14	
Me_{18}	1.56, s, 3H	15.5 (q)	H7, H12		H7, H12
Me_{19}	1.56, s, 3H	15.6 (q)	H5	H3, H6α	H5
Me_{20}	1.56, s, 3H	15.3 (q)	H1	H2	H1
1′	4.45, d, 1H, (7.8)	96.7 (d)	H2′, H3′	Me16, Me17, H3′, H5′	H2′, H5′
2′	3.41, t, 1H, (8.7)	74.5 (d)	H1′, H3′, H4′	H4′	H1′, H3′
3′	3.68, t, 1H, (9.3)	74.7 (d)	H1′, H2′, H4′	H1′	H1′, H2′, H4′
4′	4.87, t, 1H, (9.9)	70.9 (d)	H2′, H3′, H5′, H6′a,b	H2′	H3′, H5′, H6′a,b
5′	3.59, ddd, 1H, (2.7,6.0,9.7)	71.6 (d)	H4′, H6′a,b	H1′, H6′a,b	H1′, H3′, H4′, H6′b
6′a	4.10, dd, 1H, (2.4,12.0)	62.8 (t)	H4′, H5′, H6′b	H5′, H6′b	H4′
6′b	4.19, dd, 1H, (6.0, 12.0)		H4′, H5′, H6′a	H5′, H6′a	H4′
4′-OAc	2.12, s, 3H	20.9 (q)			
		170.6 (s)			H4′, 4′-OAc
6′-OAc	2.06, s, 3H	20.7 (q)			
		170.7 (s)			H6′a, 6′-OAc
2′-OH	2.38, br s,1H, exchangeable[e]				
3′-OH	2.65, br s,1H, exchangeable[e]				

[a] The ^{1}H NMR spectrum was recorded at 500 MHz in $CDCl_3$ at room temperature. Assignments were aided by spin-decoupling experiments. Chemical shifts are given in δ units downfield from Me_4Si. [b] The ^{13}C NMR spectrum was recorded in $CDCl_3$ at 125 MHz. Numbers of attached protons were determined by DEPT experiments. Assignments were aided by HMQC and HMBC experiments. [c] ^{1}H−^{1}H COSY, RCT COSY, and 2D-PSNOESY were performed in $CDCl_3$ at 300 MHz. [d] The HMBC spectrum was recorded at 500 MHz in $CDCl_3$. Protons correlated to carbon resonances in ^{13}C column. Parameters were optimized for $J_{CH} = 6$ and 8 Hz. [e] Signals within a column may be reversed.

matography followed by silica gel chromatography and normal-phase HPLC of the hexane extracts gave (+)-α-muurolene as a major metabolite (0.7% based on dry wt)[17] and calyculaglycosides A−C (**1**−**3**). Calyculaglycoside B (**2**) was the major component, comprising 0.14% of the lipid extract, while calyculaglycosides A and C represented less than 0.02% of the organic extract.

Results and Discussion

Calyculaglycoside B (**2**) was isolated as a colorless oil whose data from high-resolution mass and ^{13}C NMR spectrometry (Table 1) established a molecular formula of $C_{30}H_{48}O_8$. The ^{1}H NMR spectrum (Table 1) contained two methyl signals at δ 2.06 (3H, s) and 2.12 (3H, s) and two carbon signals at δ 170.7 (s) and 170.6 (s) in the ^{13}C NMR spectrum which, together with an intense infrared absorption at 1746 cm^{-1}, suggested that **2** was a diacetate. While the presence of five other methyl resonances [three overlapped at 1.56 (9H, s) ppm and two at 1.22 (6H, s) ppm] in addition to the acetate methyls was consistent with a diterpenoid carbon skeleton, this observation did not correlate with the molecular formula, indicating a larger molecule. A ^{13}C NMR resonance at δ 96.7 (d) assigned to a sugar anomeric carbon and five carbons in the region between δ 62−75 suggested that **2** was a hexose glycoside. The presence of an acetal and the numerous oxygenated carbons in **2**, together with the molecular formula indicating a 10-carbon fragment in excess of a diterpenoid molecule, were strong indications that calyculaglycoside B contained a diacetylated hexose sugar. A reevaluation of the mass spectral fragmentation pattern of **2**, showing $C_{20}H_{32}$ (m/z 272) [$M^+ - (C_6H_{10}O_6 + 2Ac)$; intensity = 54%] and $C_{10}H_{15}O_7$ (m/z 247) [diacetylated sugar unit; intensity = 20%] as prominent peaks, supported this assignment. A ^{1}H NMR COSY experiment, which revealed an isolated spin system of

(15) Specimens of *Eunicea sp*., under the name Sánchez USNM 97733, are on deposit in the octocoral collection, Smithsonian Institution, Washington DC under the curatorship of Dr. Frederick M. Bayer. Additional voucher specimens (ICN-MHN-CO-098) are on deposit at the Instituto de Ciencias Naturales-Museo de Historia Natural, Universidad Nacional de Colombia.

(16) *Eunicea sp*. is an undescribed species belonging to the subgenus *Euniceopsis* with a characteristic strongly armed anthocodial crown and reduced clubs sclerites in the external layer. The external appearance of *Eunicea sp*. resembles *E. fusca* Duchassaing & Michelotti, while their sclerites and anthocodial crown resemble *Eunicea calyculata* (Ellis & Solander) forma *coronata* Bayer. However, *Eunicea sp*. showed notable differences in its calyx architecture and arrangement besides the presence of a characteristic, colorless and coarse, capstans sclerites uncommon in other *Eunicea* species. Also, this species has been observed in simpatry at several Colombian Caribbean reefs. *Eunicea sp*. forms individual colonies of up to 1 m in height and profusely branched, while *E. fusca* is generally below 0.4 m in height forming a dense bed of asexual fragments. On the other hand, a phylogenetic cladistic analysis revealed an ample dichotomous separation consistent with other species and several auto-apomorphies, in both external and spicular characters, clear evidence of a valid species.

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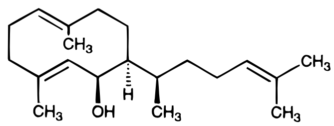
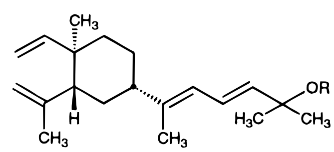
seven protons in the region of δ 4.9−3.4, confirmed that **2** was a hexose glycoside and confidently assigned all of the sugar protons (Table 1). Once the presence of the hexose residue in calyculaglycoside B (**2**) was established, attention was directed toward determining the complete structures of the aglycone and sugar components.

A combination of ^{1}H−^{1}H COSY, ^{13}C NMR, HMQC, HMBC, and HREIMS data revealed the aglycone component of calyculaglycoside B to possess a similar C_8 side chain as that found in dilophol (**4**)[12] and fuscol (**5**).[10] However, the chemical shift of the C-15 carbon [δ 81.6 (s)] and two olefinic carbon signals at δ 134.1 (s) and 125.1 (d), each showing HMBC correlations to H7 and Me-18, argued for a modified structure. Compound **2** was, therefore, proposed to possess a tertiary ether linkage at C-15 and one $\Delta^{11,12}$ trisubstituted olefin with *E* geometry on the basis of the shielded methyl carbon resonance at δ 15.5. Two prominent ion fragments at *m/z* 109 (C_8H_{13}) and 69 (C_5H_9) confirmed the existence of the modified C_8 side chain depicted in structure **2**.



dilophol (**4**)



fuscol (**5**) R= H
fuscoside B (**6**) R= α-D-arabinopyranose

The remaining components of the diterpenoid aglycone were also identified by interpretation of combined spectral data. Consideration of the molecular formula and mass spectrometry data revealed that the remaining diterpenoid component had a formula of $C_{12}H_{19}$ (*m/z* 163; intensity = 16%). Since there are four olefinic carbons remaining in the ^{13}C NMR spectrum [δ 125.7 (d), 125.8 (d), 133.1 (s), and 133.5 (s)], the C_{12} component must be a monocyclic diene system as found in dilophol (**4**). The combination of ^{13}C NMR, COSY NMR, HMQC, HMBC, and extensive interpretation of the HREI mass spectral data (Scheme S1, see Supporting Information) confirmed this contention.

Through the use of ^{1}H−^{1}H COSY, HMQC, and HMBC experiments, the sugar in **2** was identified as diacetylated glucose in a β-pyranoside configuration.[18] The low-field shifts of the C4′ proton (δ 4.87) and C6′ (δ 4.10 and 4.19) protons indicated that the acetate esters were at the sugar C4′ and C6′ positions. Relative stereochemistry of the saccharide was determined by NOE experiments and coupling constants (Table 1). The absolute configuration of glucose was D as determined by chiral GC-MS for the acid-hydrolysate. Results from these experiments demonstrated that the hexose portion of calyculaglycoside B (**2**) was a 4′,6′-di-*O*-acetyl-β-D-glucopyranose. An HMBC correlation between the anomeric proton at 4.45 ppm (H1′) to a carbon at 81.6 ppm (s, C15) connected the monosaccharide to the diterpene system. The strong NOE response between H1′ and the geminal Me-16/Me-17 pair confirmed this spatial relationship and yielded the final structure, **2**.

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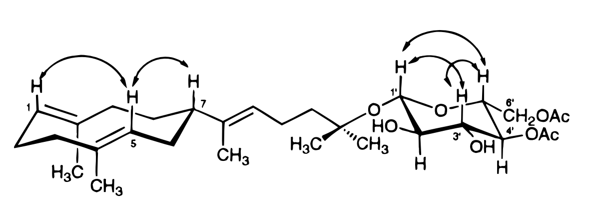

2a

Figure 1. AM1-optimized model for the major conformation of calyculaglycoside B together with selected NOE correlations.

The relative stereochemistry about the 1,5-cyclodecadiene ring moiety of calyculaglycoside B (**2**) was resolved by a combination of NOE and coupling constant data supported by distance calculations using the AM1 semiempirical method.[19] Unusual features of both the ^{13}C and ^{1}H NMR spectra were the exceptionally high resolution of all the signals and also, in the case of the ^{13}C NMR spectral data, the great stability of signal height. These observations together with the fact that only one set of signals were observed at 27 °C suggested that the 10-membered ring in **2** is dominated by a single conformation. The dominance of one conformation of **2** in solution is supported by the relative large differences in the chemical shifts, coupling constants, and NOEs of the diastereotopic methylene proton pairs H6αβ and H8αβ (Table 1). Viewing the literature with these facts led to the realization that calyculaglycoside B was quite unique, from the conformational stability point of view, since other authors had described great conformational mobility, on the NMR time-scale, for related germacrane-type diterpenoids such as dilophol (**4**).[20] The coupling constants and NOEs between any pairs of protons depicted in Table 1 (300 MHz) are interpretable only when the conformation and relative configuration of the side chain substituent are as illustrated in **2a** (Figure 1). Geometry optimization showed that the most stable conformation was exactly the same as **2a**, and the free energy difference between **2a** and the next favorable conformation was *ca*. 4.6 kcal/mol. In conclusion, the combination of two factors determine the single conformation of **2**: the 10-membered carbocycle is so small that the two endocyclic double bonds must be placed perpendicularly to an averaged plane of the 10-membered ring and the relative stereochemistry at C7 with H7 in the β-pseudoaxial position and the long side chain in the α-pseudoequatorial position. These orientations bring H5 within 2.25 and 2.32 Å, respectively, of the H7β and H1 protons, in accord with the observed NOE (all distance estimates come from molecular modeling studies as discussed above). Hence, the relative configuration of the aglycone component of calyculaglycoside B (**2**) is 7*S**. The absolute configuration of the asymmetric carbon center C7 could not, however, be determined from the experimental data. Several attempts to isolate the free aglycone by HCl hydrolysis for subsequent chemical derivatization followed by single crystal X-ray diffraction analysis resulted in unexpected decomposition of the aglycone. Hence, because limited sample size precluded continued attempts, the absolute

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stereochemistry of calyculaglycoside B (**2**) at C7 could not be established. In the present work the absolute stereochemistry shown in structures **1**-**3** is inferred; the enantiomers drawn have been chosen arbitrarily to conform with the absolute configuration of fuscol (**5**) at that position.[21]

Calyculaglycosides A (**1**) and C (**3**) were isolated as colorless oils which were also analyzed for $C_{30}H_{48}O_8$ by high-resolution mass and ^{13}C NMR spectrometry. Spectral data from these compounds were highly comparable with those from the diacetate **2**. The chemical shifts of many signals in the ^1H and ^{13}C NMR spectra of **1** and **3** were very similar to those from calyculaglycoside B (**2**) (Tables 1 and 2). Using a combination of ^1H NMR COSY, HMQC, and HMBC data all of the proton-bearing carbons, their protons, and the quaternary carbons in calyculaglycosides A and C were precisely matched. Thus, it was concluded with confidence that **1** and **3** have the same diterpenoid aglycone component found in **2** and that the minor differences seen in their NMR spectra were due to a distinct sugar residue. The highly comparable HREI mass spectral data confirmed that diacetates **1**−**3** indeed possess identical diterpenoid aglycones.

Although interpretation of ^1H NMR data in $CDCl_3$ for the sugar unit in calyculaglycoside A (**1**) was sometimes hampered by severe overlapping and peak broadening of the oxymethines, this problem could be solved by measuring the NMR spectra in benzene-d_6. Chemical shifts for the protons at C3′ and C6′ indicated that the hydroxyl groups on these carbons were acetylated. On the other hand, the low-field shifts of the CH_2-6′ protons (4.11 and 4.13 ppm) and that of the H4′ proton (5.30 ppm) in calyculaglycoside C (**3**) $CDCl_3$ solution indicated that the acetate esters were at the sugar C4′ and C6′ positions. In each case, interpretation of ^1H NMR and NOESY data allowed assignment of a diacetylated galactopyranose unit with an axial anomeric proton. The absolute stereochemistry of the galactose residues was determined to be D by chiral GC-MS analysis. These results demonstrated conclusively that the hexose components of **1** and **3** were 3′,6′-di-*O*-acetyl-β-D-galactose and 4′,6′-di-*O*-acetyl-β-D-galactose, respectively.

Calyculaglycosides **1**−**3** (CLG-A, CLG-B, and CLG-C) represent an important new class of antiinflammatory compounds. The topical antiinflammatory properties of CLG-B (**2**) were observed during *in vivo* assays against bee venom PLA_2, arachidonic acid (AA), and croton oil-induced inflammation in mouse ears.[22] CLG-B doses below 0.3 μM/ear were not effective against bee venom PLA_2-induced edema, indicating **2** may act at a level subsequent to the formation of AA. In contrast, CLG-B reduced arachidonic acid (AA)-induced mouse ear inflammation by 92% at a testing dose of 125 μg/ear, indicating CLG-B may be an inhibitor of the 5-lipoxygenase (5-LO) pathway. CLG-B also inhibited edema induced by croton oil (a mixture of phorbol esters) at a level comparable with indomethacin (77% and 43% inhibition at 125 and 60 μg CLG-B/ear, respectively) suggesting that CLG-B may also inhibit PGE_2 synthesis from arachidonic acid via the cyclooxygenase (COX) pathway. For calibration purposes, indomethacin reduced edema by 76% at 250 μg/ear. In contrast to aspirin, ibuprofen, or indomethacin, CLG-B appears to act by a different mechanism, as it did inhibit arachidonic acid (AA)-induced inflammation. These preliminary observations led us to hypothesize that **2** is acting nonselectively to inhibit both the 5-LO and COX pathways.

The effects of calyculaglycosides A−C (**1**−**3**) tested *in vitro* at 10^{-9}, 10^{-7}, and 10^{-5} M on one enzyme activity and on several inflammation assays are indicated in Table S3 (see Supporting Information). Results are expressed as a percentage of inhibition and are the means of two determinations. Calyculaglycosides A (**1**) and C (**3**) showed weak to no inhibition of the *Naja naja naja* phospholipase A_2 at all three concentrations. On the other hand, CLG-A appears to have a dramatic effect on PGE_2 synthesis and release: at 10^{-5} M, **1** greatly activates PGE_2 secretion by almost 900% with little or no activation of the cyclooxygenase pathway detected at lower concentrations. Interestingly, CLG-B showed 40% inhibition of LTB_4 secretion at 10^{-5} M, suggesting again that **2** may act as an inhibitor of the 5-lipoxygenase pathway. Considering that CLG-A and CLG-B are structurally similar, these observations suggest that inhibition of the 5-LO pathway may consequently lead to activation of the COX pathway. At concentrations below 10^{-5} M, CLG-B and CLG-C had negligible effects on Il-1β and TNFα release, respectively, showing again that the mode of action of these antiinflammatory agents is based on their ability to mediate AA metabolism and not by blocking the release of these cytokines by lymphocytes and macrophages. While further studies directed at unraveling the exact mechanism of action of the calyculaglycosides **1**−**3** are needed, these marine natural products have already demonstrated efficacy in several models of inflammation and may eventually prove to be clinically useful against certain disease states. [23]

The NCI *in vitro* primary disease-oriented antitumor screen was also used to ascertain the cytotoxic properties of two of the diterpene-glycosides described here. Of the two compounds, calyculaglycoside B (**2**) was the more potent, with concentrations of 10^{-4}−10^{-5}M eliciting strong differential responses at the LC_{50} level from nearly all the ovarian cell lines and from several of the renal, prostate, and colon cancer cell lines. Calyculaglycoside C (**3**) gave a similar pattern but required higher concentrations. Thus, the calyculaglycosides and related metabolites may, therefore, be of interest for *in vivo* evaluation in appropriate xenograft tumor models.

Experimental Section

General Experimental Procedures. ^1H NMR, ^{13}C NMR, HMQC, and HMBC spectra were recorded on a Bruker Avance DRX-500 spectrometer. ^1H−^1H COSY, RCT COSY, and 2D-NOESY spectra were recorded on a General Electric QE-300 or Bruker DPX-300 spectrometers. ^1H-NMR chemical shifts

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(23) In spite of the close structural relationship and comparable potency between the calyculaglycosides and some fuscosides (*i.e.* fuscoside B), the former antiinflammatory agents appear to act by a different mechanism. For instance, CLG-B (**2**), like fuscoside B (**6**), did not inhibit bee venom PLA_2 and effectively inhibited leukotriene synthesis and phorbol myristate acetate (PMA)-induced edema in mouse. Yet, in contrast to CLG-B, fuscoside B had negligible effects on PGE_2 synthesis and was not effective against (AA)-induced inflammation in mouse ears at doses below 500 μg/ear (see Jacobs, R. S.; Bober, M. A.; Pinto, I.; Williams, A. B.; Jacobson, P. B.; de Carvalho, M. S. In *Marine Biotechnology. Pharmaceutical and Bioactive Natural Products*; Attaway, D. H.; Zaborsky, O. R., Eds.; Plenum Press: New York, 1993; Vol. 1, pp 77−99 and reference 11).

Table 2. ^{1}H and ^{13}C NMR Spectral Data of Calyculaglycosides A (1) and C (3)

	calyculaglycoside A (**1**)[a,b]		calyculaglycoside C (**3**)[a,b]	
position	^{1}H intgr mult (*J*)	^{13}C	^{1}H intgr mult (*J*)	^{13}C
1	4.94, 1H, br t (6.6)	125.7 (d)	4.93, 1H, br t (6.7)	125.7 (d)
2	2.10, 2H, m	24.7 (t)	2.13, 2H, m	24.7 (t)
3	2.07, 2H, m	38.9 (t)	2.08, 2H, m	38.8 (t)
4	–	133.5 (s)	–	133.6 (s)
5	5.06, 1H, br t (6.3)	125.8 (d)	5.07, 1H, br t (6.6)	125.8 (d)
6α	2.14, 1H, m	28.2 (t)	2.17, 1H, m	28.2 (t)
6*β*	1.87, 1H, m (6.9, 7.5, 15.3)		1.88, 1H, m (7.3, 7.5,15.1)	
7	1.45, 1H, m (4.0, 7.4, 11.3)	47.8 (d)	1.47, 1H, m (3.8, 7.5, 11.3)	47.7 (d)
8α	1.22, 1H, m	28.2 (t)	1.23, 1H, m	28.2 (t)
8*β*	1.62, 1H, m (4.2)		1.61, 1H, m	
9	1.99, 2H, m	39.4 (t)	2.03, 2H, m	39.4 (t)
10	–	133.1 (s)	–	133.1 (s)
11	–	134.2 (s)	–	134.1 (s)
12	4.99, 1H, br t (6.4)	125.0 (d)	5.00, 1H, br t (6.4)	125.0 (d)
13	2.06, 2H, m	24.0 (t)	2.08, 2H, m	24.0 (t)
14	1.97, 2H, m	37.9 (t)	2.00, 2H, m	37.8 (t)
15	–	81.7 (s)	–	81.6 (s)
16	1.22, 3H, s[c]	23.8 (q)[c]	1.22, 3H, s[c]	23.8 (q)[c]
17	1.23, 3H, s[c]	24.8 (q)[c]	1.23, 3H, s[c]	24.8 (q)[c]
18	1.54, 3H, s	15.6 (q)	1.56, 3H, s	15.6 (q)
19	1.54, 3H, s	15.6 (q)	1.56, 3H, s	15.6 (q)
20	1.56, 3H, s	15.3 (q)	1.56, 3H, s	15.3 (q)
21				
1′	4.51, 1H, d (7.8)	97.1 (d)	4.44, 1H, d (7.8)	97.2 (d)
2′	3.45, 1H, t (9.0)	72.2 (d)	3.71, 1H, dd (7.6, 9.6)[d]	72.0 (d)
3′	4.93, 1H, dd (3.3, 9.3)	78.0 (d)	3.45, 1H, dd (3.6, 9.7)[d]	72.3 (d)
4′	3.49, 1H, br s	69.7 (d)	5.30, 1H, dd (0.9, 3.6)	69.1 (d)
5′	3.50, 1H, br s	73.9 (d)	3.81, 1H, dt (1.2, 6.9)	70.8 (d)
6′a	4.34, 1H, br s	63.5 (t)	4.11, 1H, br s	62.1 (t)
6′b	4.34, 1H, br s		4.13, 1H, br s	
3′ or 4′-OAc	2.16, 3H, s	21.0 (q)	2.14, 3H, s	20.8 (q)
		172.4 (s)		171.1 (s)
6′-OAc	2.08, 3H, s	20.8 (q)	2.03, 3H, s	20.7 (q)
		171.3 (s)		170.5 (s)
OH's	3.06, 2H, br s[e]		2.57,1H, br s;[e] 2.63,1H, br s[e]	

[a] ^{1}H NMR spectra were recorded at 300 MHz in $CDCl_3$ at room temperature. Assignments were aided by ^{1}H−^{1}H COSY, RCT COSY, 2D-PSNOESY, and spin-decoupling experiments. J values are reported in hertz, and chemical shifts are given in δ units (downfield from Me_4Si). [b] ^{13}C NMR spectra were recorded in $CDCl_3$ at 75 MHz. Numbers of attached protons were determined by DEPT experiments. Assignments were aided by HMQC and HMBC experiments. [c] Signals within a column may be reversed. [d] Peak broadening in $CDCl_3$ did not allow for an assignment of multiplicity and J data. Therefore, these values are reported in C_6D_6 solution. [e] D_2O exchanged.

were recorded with respect to internal Me_4Si and ^{13}C-NMR chemical shifts are reported in ppm relative to $CDCl_3$ (77.0 ppm). Column chromatography (CC) was carried out on Si gel (35−75 mesh) and HPLC was performed using a 10 μm silica gel Partisil 10 semipreparative column (9.4 mm × 50 cm). All solvents used were either spectral grade or were distilled from glass prior to use. Unless otherwise noted, materials were obtained from commercial suppliers and were used as provided. The enantiomeric sugars and *N*-(heptafluorobutyryl)imidazole were purchased from Sigma.

Collection and Extraction. Minced and freeze-dried specimens of *Eunicea sp.*[15,16] (2.1 kg) collected near Santa Marta Bay, Colombia, in March, 1996, were extracted with $CHCl_3$-MeOH (1:1) (8 × 1 L). After filtration, the extract was evaporated to yield a residue (170.5 g) that was suspended in water and then partitioned between *n*-hexane (6 × 2.5 L), $CHCl_3$ (8 × 4 L), and *n*-BuOH (5 × 1.5 L). The hexane extract was concentrated to yield 72.0 g of a green oil which, after filtration, was fractionated by size exclusion chromatography on a Bio-Beads SX-3 column with toluene as eluant. The combined portions (TLC guided) were concentrated to obtain three main fractions, the last one of which consisted of (+)-α-muurolene (14.5 g, 0.7% based on dry wt).[17] The remaining two fractions [fractions A (31.7 g) and B (24.3 g)] were concentrated and evaluated by NMR. A portion of fraction B (*ca.* 7.0 g) was chromatographed over silica gel (750 g) with 20% hexane−EtOAc and fractionated roughly into subfractions 1 through 16. Calyculaglycoside-A (**1**) (15.9 mg, 0.08% of the total extract) was isolated from subfraction-11 (91.1 mg) after HPLC using 95:5 hexane/2-propanol. Subfraction 14 (215 mg), which consisted mainly of an intense red pigment, was dissolved in $CHCl_3$ and, after repeated CC on Si gel using 95:5 $CHCl_3$/acetone and then hexane/acetone mixtures, yielded 30.0 mg of calyculaglycoside-B (**2**) (0.14% of the extract) and 13.4 mg of calyculaglycoside-C (**3**) (0.06% of the extract).

Calyculaglycoside A (1): isolated as a colorless oil: $[\alpha]^{24}_D$ +9.2° (*c* 0.6, $CHCl_3$); UV λ_{max} (MeOH) nm (log ϵ) 214 (3.88); IR (neat) 3445, 2922, 2872, 2853, 1748, 1733, 1456, 1373, 1259, 1242, 1163, 1090, 1037, 978 cm^{-1}; HRFABMS *m*/*z* $[M + Na]^+$ obsd 559.32474, $C_{30}H_{48}O_8Na$ required 559.32469; HREIMS *m*/*z* $[M\text{-}C_{10}H_{14}O_7 - H_2O]^+$ obsd 272.25137, $C_{20}H_{32}$ required 272.25040; low-resolution MS *m*/*z* (relative intensity) 272 (55), 257 (30), 247 (3), 229 (19), 201 (11), 189 (25), 175 (19), 163 (13), 162 (14), 161 (32), 149 (26), 148 (27), 147 (32), 137 (16), 136 (39), 135 (45), 134 (28), 133 (37), 127 (11), 123 (34), 122 (35), 121 (93), 119 (40), 109 (36), 107 (78), 93 (100), 91 (35), 78 (42), 72 (50), 68 (87); ^{1}H and ^{13}C NMR (see Table 2).

Calyculaglycoside B (2): isolated as a colorless oil: $[\alpha]^{26}_D$ +11.4° (*c* 0.5, $CHCl_3$); UV λ_{max} (MeOH) nm (log ϵ) 210 (3.67); IR (neat) 3436, 2960, 2923, 2854, 1746, 1437, 1369, 1239, 1165, 1087, 1062, 1030, 902, 802 cm^{-1}; HRFABMS/LiCl *m*/*z* $[M + Li]^+$ obsd 543.34964, $C_{30}H_{48}O_8Li$ required 543.35092; HREIMS *m*/*z* $[M\text{-}C_{10}H_{16}O_7]^+$ obsd 288.24542, $C_{20}H_{32}O$ required 288.24532, $[M - C_{10}H_{14}O_7 - H_2O]^+$ obsd 272.24970, $C_{20}H_{32}$ required 272.25040; low-resolution MS *m*/*z* (relative intensity) 288 (4), 272 (54), 257 (15), 247 (20), 229 (13), 217 (10), 203 (12), 189 (20), 187 (30), 175 (14), 163 (16), 162 (13), 161 (25), 149 (29), 148 (26), 137 (26), 136 (49), 135 (45), 134 (22), 133 (25), 127 (28), 123 (40), 122 (39), 121 (62), 119 (26), 109 (36), 107 (56), 93 (71), 81 (100), 79 (31), 73 (25), 69 (86), 68 (58), 67 (41); ^{1}H and ^{13}C NMR (see Table 1).

Calyculaglycoside C (3): isolated as a colorless oil: $[\alpha]^{24}_D$ +7.8° (*c* 0.5, $CHCl_3$); UV λ_{max} (MeOH) nm (log ϵ) 212 (3.67); IR (neat) 3458, 2952, 2922, 2870, 2853, 1747, 1734, 1456, 1437, 1370, 1259, 1238, 1167, 1121, 1080, 1033, 947, 908, 842, 803 cm^{-1}; HRFABMS *m*/*z* $[M + Li]^+$ obsd 543.34963, $C_{30}H_{48}O_8Li$

required 543.35092; HREIMS *m/z* [M − $C_{10}H_{16}O_7$]$^+$ obsd 288.24684, $C_{20}H_{32}O$ required 288.24532, [M − $C_{10}H_{14}O_7$ − H_2O]$^+$ obsd 272.24767, $C_{20}H_{32}$ required 272.25040; low-resolution MS *m/z* (relative intensity) 288 (2), 272 (48), 257 (14), 247 (20), 229 (13), 217 (10), 203 (12), 189 (20), 187 (19), 175 (14), 163 (16), 162 (13), 161 (27), 149 (29), 148 (26), 147 (25), 137 (29), 136 (48), 135 (46), 134 (20), 133 (23), 127 (21), 123 (40), 122 (37), 121 (64), 119 (27), 115 (17), 109 (38), 107 (52), 105 (26), 95 (52), 93 (67), 91 (26), 83 (21), 81 (100), 79 (34), 69 (99), 68 (59), 67 (47); ^{1}H and ^{13}C NMR (see Table 2).

Chiral GC-MS Analysis of Acid Hydrolysates.[24] A 0.5 mg portion of each glycoside was dissolved in 1 N HCl (0.2 mL) and heated at 48−50 °C for 3 h. The reaction mixture was evaporated in a stream of nitrogen, stored under vacuum, suspended in *N*-(heptafluorobutyryl)imidazole (0.1 mL), heated for 30 min at 60 °C with occasional mixing, and evaporated in a stream of nitrogen. The residue was dissolved in CH_2Cl_2 (0.1 mL), and the resulting, clear solution was chromatographed directly on a Chirasil-Val (Alltech) column. The oven temperature was maintained for 3 min at 60 °C and raised to 200 °C at 4 °C/min. Retention times for the authentic monosaccharide residues after simultaneous treatment with 1 N HCl and worked up in the same manner (min): D-galactose (15.04), L-galactose (15.28), D-glucose (14.65), L-glucose (15.01). Retention times (min) of GC peaks in the acid hydrolysate of: calyculaglycoside A (**1**): 15.04 (D-galactose); calyculaglycoside B (**2**): 14.61 (D-glucose); calyculaglycoside C (**3**): 15.02 (D-galactose).

Acknowledgment. The assistance of F. M. Ablanque and H. Castellanos in specimen collection is gratefully acknowledged. We extend our sincere appreciation to Dr. Marie C. Bodinier and Dr. Valerie Hamond (CEREP) for the *in-vitro* enzyme activity and inflammation assays, to Dr. Mirtha Llanio (CEBIMAR) for the *in-vivo* antiinflammatory activity tests, and to Dr. Edward Sausville (NCI) for the *in-vitro* anticancer screening on human cells. HRFABMS spectral determinations were performed by the Midwest Center for Mass Spectrometry at the University of Nebraska−Lincoln, a National Science Foundation Regional Facility (Grant No. CHE8211164). We gratefully appreciate the taxonomic assignment provided by Dr. Frederick M. Bayer, Smithsonian Institution, Washington, DC. This study was supported by the NSF-EPSCoR (Grant No. R118610677), NIH-MBRS (Grant No. S06RR08102-17), NIH-MARC U*STAR (Grant No. 5T34GM07821-17), NSF-MRCE (Grant No. R11-8802961) and ATLANTEA (Grant No. RRP-61-II-1995−96) Programs of the University of Puerto Rico at Río Piedras.

Supporting Information Available: Copies of the HREIMS and IR spectra as well as ^{1}H, ^{13}C, and HMBC NMR spectra of **2** plus Scheme S1 and Table S3 (10 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO9704725

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